

## Original Article



# Real-time Reverse Transcription PCR-based SARS-CoV-2 Detection in Khwaja Yunus Ali Medical College Hospital, Enayetpur, Sirajganj, Bangladesh

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### Abstract

**Background:** Coronavirus disease 2019 (COVID-19) is a global pandemic declared on 11 March 2020 by World Health Organization (WHO). It is a highly pathogenic viral infection caused by SARS-CoV-2 which has caused global health concern. The emergence of SARS-CoV-2 infection has triggered a global need for accurate diagnostic assays. Detection of viral RNA in clinical specimens is the hallmark of diagnosis. **Objectives:** The aim of present study was to determine the prevalence of SARS-CoV-2 infection in some districts of North Bengal area of Bangladesh according to age, sex and area distribution. **Materials and Methods:** This cross sectional descriptive study was carried out in the Department of Laboratory Services, KYAMCH, Enayetpur, Sirajganj, Bangladesh. Real-Time reverse transcription Polymerase chain reaction assay was used for detection of virus. **Results:** A total of 1760 cases in the month from March to May 2021 were tested, among which 310 (17.6%) cases were detected positive for SARS-CoV-2. Out of 310 positive cases 211 (68.06%) were male and 99 (31.94%) were female. Maximum positive cases were from Sirajganj followed by Pabna, Natore, Naogaon, Bogura, Gaibandha districts and some parts of Rajshahi district respectively. **Conclusion:** This is the first study for genetic detection of SARS-CoV-2 done in KYAMCH, Enayetpur, Sirajganj. Strategies of using rRT-PCR for detection of SARS-CoV-2 may facilitate early and confirmatory diagnosis of COVID-19

**Key words:** COVID-19, SARS-CoV-2, Pandemic, Molecular, rRT-PCR, Coronavirus

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### Introduction

In late December 2019, the world came to know about an unknown threat caused by a pathogen with unidentified etiology originating from a seafood market in Wuhan in Hubei Province, China, and the Chinese Center for Disease Control and Prevention (CCDC) proclaimed it as novel coronavirus pneumonia (NCP).<sup>1</sup> The virus now known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is which belongs to the Coronaviridae family.<sup>2</sup> Different from both MERS-CoV and SARS-CoV, 2019-nCoV is the seventh member of the family of coronaviruses that infect humans.<sup>3</sup> Coronavirus disease 2019

(COVID-19) is a global pandemic declared on 11 March 2020 by World Health Organization (WHO). According to the report of WHO on 25 June, 2021 the virus has spread to at least 220 countries with more than 17,96,86,071 confirmed cases and 38,99,172 confirmed deaths have been recorded, with massive global increases in the number of cases daily.<sup>4</sup> The virus has likely spread too widely to make eradication a possibility, at least without an effective vaccine, better disease surveillance and major safeguards on the way humans come into contact with wild animals. The high transmissibility, long incubation period and the containment of the disease is extremely difficult.<sup>5</sup> The

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virus was confirmed to have spread to Bangladesh in March 2020. The first three known cases were reported on 8 March 2020 by the country's epidemiology institute, IEDCR. Since then, the pandemic has spread day by day over the whole nation and the number of affected people has been increasing.<sup>6</sup> Bangladesh faces significant challenges in combating COVID-19 as it is a densely populated country and also houses a million stateless Rohingya refugees in sprawling refugee camps that are conducive to the spread of epidemics.<sup>7,8</sup>

Usually, this newly identified SARS-CoV-2 has an incubation period of 2-7 days. Symptoms of COVID-19 are variable, ranging from mild symptoms to severe illness. Common symptoms include headache, loss of smell and taste, nasal congestion and runny nose, cough, muscle pain, sore throat, fever, diarrhea, and breathing difficulties. People with the same infection may have different symptoms, and their symptoms may change over time. Three common clusters of symptoms have been identified: one respiratory symptom cluster with cough, sputum, shortness of breath, and fever; a musculoskeletal symptom cluster with muscle and joint pain, headache, and fatigue; a cluster of digestive symptoms with abdominal pain, vomiting and diarrhea.<sup>9,10,11</sup> SARS-CoV-2 is mainly transmitted via the respiratory route when people inhale droplets and particles that infected people release as they breathe, talk, cough, sneeze or sing. Infection can occur over longer distances, particularly indoors.<sup>12,13</sup> Infectious particles range in size from aerosols that remain suspended in the air for long periods of time to larger droplets that remain airborne or fall to the ground.<sup>14</sup>

Viruses constantly change through mutation. When a virus has one or more new mutations it's called a variant of the original virus. Currently, several variants of the virus (SARS-CoV-2) that causes COVID-19 are creating concern in different countries of the world. In December 2020, an unexpected rise in reported COVID-19 cases was attributed to the emergence of the new SARS-CoV-2 variants 501Y.V1 (B.1.1.7) in the UK and 501Y.V2 (B.1.351) in South Africa.<sup>15</sup> In January 2021, another set of mutations in a new P.1 (501Y.V3) lineage has been identified in Manaus, Brazil.<sup>16</sup> SARS-CoV-2 Delta variant, also known as lineage B.1.617.2, was first detected in India in late 2020. The World Health Organization (WHO) named it the Delta variant on 31 May 2021.<sup>17</sup>

Accurate and early detection of SARS-CoV-2 is vital to decrease the risk of transmission by rapidly enabling isolation and contact tracing. The value of diagnostic methods depends on the type of test, the time to get the results, testing accuracy and the required resources for testing.<sup>18,19</sup> Nucleic acid (NA) testing is reported to be essential for the early diagnosis and confirmation of SARS-CoV-2 infection. Among them Real-time reverse transcription polymerase chain reaction (rRT-PCR) is the most popular and the "gold standard" testing method for the detection of SARS-CoV-2.<sup>20</sup> In this method, the SARS-CoV-2 RNA is first reverse transcribed into cDNA, and specific gene fragments within the cDNA are amplified using target-specific primers. The fluorescence signal, which

represents the copy number of the target sequence, can be easily detected during the amplification process.<sup>21</sup>

## Materials and Methods

This cross-sectional descriptive study was carried out in the Department of Laboratory Services (Microbiology), Khwaja Yunus Ali Medical College Hospital, Enayetpur, Sirajganj, Bangladesh from March to May, 2021. A total of 1760 nasopharyngeal and oropharyngeal swabs were taken from suspected patients of Sirajganj, Pabna, Natore, Naogaon, Bogura, Gaibandha districts and some parts of Rajshahi division, Bangladesh irrespective of their age and sex. Patients having either history of fever, cough, respiratory distress, travel history or contact history with migrant populations living in COVID-affected country within two weeks were included in this study.

## Specimen collection and shipment

All specimens (nasopharyngeal and oropharyngeal swabs) collected for laboratory investigations were regarded as potentially infectious. Trained health care personnel following adequate standard operating procedures (SOP) for infection prevention and control guidelines. Specimens were shipped to the laboratory as soon as possible after collection following correct handling during transportation. Specimens were immediately processed. Specimens tested within 24 hours were stored at 4°C temperature. Specimens that were tested after 24 hours were stored at -200°C. Repeated freezing and thawing of specimens were avoided to get better results. National guidelines on laboratory biosafety were followed in all circumstances. Specimen handling for molecular testing was done in BSL-2 laboratory following appropriate biosafety practice.

## Real-Time Reverse Transcription Polymerase Chain Reaction Assay for SARS-CoV-2

Assay for detection of SARS-CoV-2 was performed by Roche LightCycler® 96 System Inc.

## Test Principle

The test is performed with RNA as starting material. The RNA is converted into cDNA with a reverse transcriptase enzyme and afterwards amplified in a PCR. The primers were designed for the selective amplification of the transcribed cDNA of the viral RdRP gene<sup>22</sup> and E gene<sup>23</sup>. The amplicons are detected with likewise SARS-CoV-2 specific fluorescent dye-labelled hydrolysis probes (TaqMan® probes). If amplicons are present, the probes are hydrolyzed by the Taq polymerase and a fluorescence signal is generated that increases proportionally with the amount of the PCR product. The fluorescence signals are measured by the optical detection unit of the real-time PCR cyclers. The test is performed in a single PCR reaction that detects the two viral genes RdRP and E and an universally expressed human housekeeping gene (Rnase P) with different fluorescent colors. The detection of Rnase P indicates the correct sampling, RNA-isolation and RT-PCR-amplification.

## Component of the diagnostic Kit

This kit is an amplification reaction reagent and contains the following components

No .	Reagent	Ingredients
1	ViroQ Enzyme	Lyophilized, contains Reverse Transcriptase, Taq Polymerase, dNTPs
2	ViroQ Solvent	Contains reconstitution buffer for the ViroQ Enzyme
3	ViroQ Mix	Contains Primer -Probe Mixture: Colour 1: Virus gene 1 (FAM) Colour 2: Virus gene 2 (HEX) Colour 3: Cell control (CFR610) (housekeeping gene)  Storage buffer
4	ViroQPos Ctrl	Positive control, dried, contains human mRNA, Virus reference RNA

## Test Procedure

### a) Safety conditions and special remarks

Special safety conditions were maintained in order to avoid contamination and false reactions. We decontaminated the working area before starting test procedures. We used gloves during whole procedures, changes gloves as required, and used new sterile pipette tips with each pipetting step (with integrated filter). Separate working areas were dedicated for pre-amplification (RNA isolation and PCR set up) and post –amplification (detection) procedures. All laboratory works were done in Class II Biological Safety Cabinet.

### b) RNA Isolation

We used Ribospin™ vRD kit for RNA isolation manufactured by Gene All Biotechnology Co., Ltd, South Korea. At first we transferred upto 300 ul sample in 1.5 ml microcentrifuge tube. Then 500 ul of buffer VL was added to the tube and lysed the sample by vortexing. We incubated the lysate for 10 minutes at room temperature. Then 700 ul of buffer RB1 was added to the lysate and mixed thoroughly by vortexing. Upto 750 ul of the mixture was transferred to a mini spin column. Centrifuge was done at  $\geq 10,000 \times g$  for 30 seconds at room temperature. 500 ul of buffer RBW was added to the mini spin column. Centrifuge was done at  $\geq 10,000 \times g$  for 30 seconds at room temperature. 500 ul of buffer RNW was added to the mini spin column. Centrifuge was done at  $\geq 10,000 \times g$  for 30 seconds at room temperature. Again centrifuge was done at  $\geq 10,000 \times g$  for an additional 1 minute at room temperature to remove residual wash buffer. Then mini spin column was transferred to a new 1.5 ml microcentrifuge tube. 30-50 ul of nuclease-free water was added to the center of the membrane in the mini spin column. Then again centrifuge was done at  $\geq 10,000 \times g$  for 1 minute at room temperature.

### c) Reagent preparation

#### ViroQ Enzyme

The enzyme mix reagent ViroQ Enzyme is lyophilized. At first we dissolved ViroQ Enzyme with 400  $\mu$ l ViroQ Solvent by pipetting up and down.

#### ViroQPos Ctrl

At first we dissolved ViroQ Positive Control with 30  $\mu$ l RNase-free H<sub>2</sub>O by pipetting up and down, allowed complete rehydration for 15 minutes and then mixed thoroughly by vortexing.

### d) PCR Amplification

We placed PCR reaction tubes into the specimen wells to the amplification equipment. Then we set up the ViroQ Positive Control, Negative Control and specimens to be tested in the corresponding sequence and input specimen name. We used FAM Channel (RNA-dependend RNA-Polymerase gene), HEX channel (E gene) to detect SARS-CoV-2 and Texas Red channel as internal control.<sup>24</sup>

Amplification program was performed with the following parameters:

Step	Time	Temperature	No. of cycles
Reverse Transcription	20 min	48°C	1 cycle
Polymerase activation	3 min	95°C	1 cycle
Denaturation	15 sec	95°C	45 cycles
Annealing + Extension	30 sec + reading	58°C	

Roche: LightCycler® 96 Real Time Cycler System was validated for the ViroQ SARS-CoV-2 kit.

**e) Interpretation of results**

The following two tables (Table I and Table II) show the interpretation of the amplification results:

Conclusion	Amplification results
SARS -CoV -2 Positive	There is typical sigmoidal shaped amplification curve detected at FAM and/or HEX channel and the amplification curve which is detected at Texas Red (CFR610)channel, Ct - values ≤ 35.
SARS -CoV -2 Negative	There is no typical sigmoidal shaped amplification curve (No Ct) or Ct >35 detected at FAM and/or HEX channel, and the amplification curve which is detected at Texas Red (CFR610) channel

FAM (RdRp gene)	HEX (E gene)	Texas Red (IC/House keeping gene)	Result
+	+	+	SARS - CoV - 2 specific RNA detected.
+	-	+	SARS - CoV - 2 specific RNA detected.
-	+	+	Beta - CoV specific RNA detected. Repeat the test with the same or a new sample.
-	-	+	SARS - CoV-2 specific RNA not detected. The sample does not contain detectable or sufficient amounts of copies (LoD) of specific RNA.
-	-	-	Invalid result due to real - time PCR inhibition or reagent failure. Repeat RNA isolation and/or testing from original sample.



**Fig 1:** Fluorescence amplification signal curve over time in Realtime thermal cycler



**Fig 2:** Amplification curves, Heat Map and Result table during interpretation of result

### Results

A total of 1760 samples were examined for detection of SARS-CoV-2 by rRT PCR from Sirajganj, Pabna, Natore, Naogaon, Bogura, Gaibandha districts and some parts of Rajshahi division, Bangladesh. Among them 310 (17.61%) were positive. Out of 310 positive cases 211 (68.06%) were male and 99 (31.94%) were female (Figure 3). Table III shows age wise distribution of patients with SARS-CoV-2 infection in the aforementioned areas. Area wise distribution of positive cases are shown in Figure 2. Maximum positive cases hailed from Sirajganj followed by Pabna, Natore, Naogaon, Bogura, Gaibandha districts and some parts of Rajshahi division respectively.

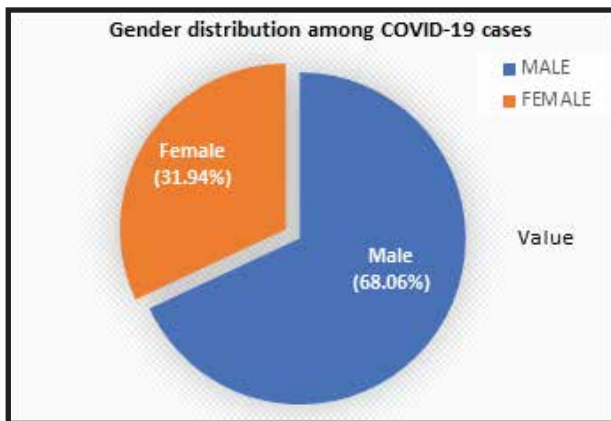


Figure 3: Distribution of patients with SARS-CoV-2 infection by sex (n=310)

Table III: Age wise distribution of patients with SARS-CoV-2 infection (n=310)

Age group (years)	Number	Percent (%)
<10	02	0.65%
11-20	12	3.87%
21-30	30	9.68%
31-40	44	14.19%
41-50	48	15.48%
51-60	81	26.13%
61-70	46	14.84%
71-80	30	9.68%
81-90	15	4.84%
91-100	02	0.65%
Total	310	100%

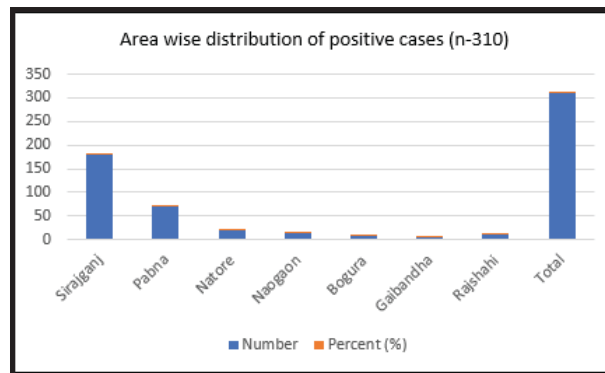


Figure 4: District wise distribution of patients with SARS-CoV-2 infection(n=310)

### Discussion

The current COVID-19 pandemic presents a serious public health crisis and a better understanding of the scope and spread of the virus would be aided by more widespread testing. Nucleic acid based tests currently offer the most sensitive and early detection of COVID-19.<sup>25</sup> However, Real-time reverse transcription polymerase chain reaction (rRT-PCR) is the “gold standard” method for the detection of SARS-CoV-2 because it has shown to be very sensitive and highly specific for accurately detecting viral genomes present, down to just one molecule of RNA.<sup>26</sup> In our present study we found 17.61% cases positive (310 positive out of 1760 cases) detected in this hospital from April to June 2021 and the cases hailed from several districts of North Bengal of Bangladesh. This is not similar with the finding of NHaque et al., 2020 in which only 7.5% cases were positive, the study done in Mymensingh Medical College Hospital and the cases from Mymensingh division, Bangladesh.<sup>27</sup> Epidemiological, phenotypic and genomic characterization of certain variants of SARS-CoV-2 have highlighted the increasing transmissibility, infectivity and antigenic escape capability of this virus. But our finding is similar with the reports published by Directorate General of Health Services: Coronavirus Update. They mentioned that the average test positivity rate in the last week of March 2021 was 17% country-wide.<sup>28</sup>

We found a greater number of males (68.06%) than females (31.94%) in these 310 positive cases of SARS-CoV-2 infection (Figure 1). Similar finding was in the study of N Haque et al., 2020 in which males were 65.9% and females were 34.1% among the total positive cases. MERS-CoV and SARS-CoV infection have also been found to infect more males than females.<sup>29,30</sup> Women, compared to men, are less susceptible to viral infections based on a different innate immunity, steroid hormones and factors related to sex chromosomes. The presence of two X chromosomes in women emphasize the immune system even if one is inactive. The immune regulatory genes encoded by X chromosome in female gender causes lower viral load levels and less inflammation than in man. In addition, women generally produce higher levels of antibodies which remain in the circulation longer. The levels of activation of the immune cells are higher in women than in men, and it is correlated with the trigger of Toll Like Receptor 7 and the

production of Interferon.<sup>31</sup> In comparison to the global perspective, the rate of infection of COVID 19 is comparatively low in Bangladesh, as 17.61% positive was found in our study and 7.5% was positive in the study of N Haque et al. 2020 despite of one of the most densely populated country (about 160 million people) in the world.<sup>27,32</sup> We know that some environmental factors, such as temperature, humidity and wind speed have a significant effect of the survival and transmission of SARS-CoV-2. The tropical monsoon climate in Bangladesh is characterized by summer heavy rainfall and high temperature with excess humidity. The range of ambient temperature in summer is 36-40°C and winter temperature is 8-15°C in Bangladesh. So, the cases and the fatality rate of COVID 19 are expected to very low in Bangladesh particularly in summer season.<sup>33</sup> The data from the current study reveals that among the positive cases 0.65% of the children were younger than 10 years. Chinese CDC estimates reveal that less than 1% of the children younger than 10 years of age were affected by the disease.<sup>34</sup> It may be possible that children had mild asymptomatic infection and were not detected or they were less likely to be infected. Maximum positive cases were found in Sirajganj district. It may be due to that this hospital is located in Sirajganj and this makes more convenience for the patients of this district to come to this hospital to be diagnosed and for treatment.

## Conclusion

The ongoing COVID-19 pandemic caused by SARS-CoV-2 and its newly emerged variants of concern are undoubtedly a matter of global health concern. rRT-PCR proved its efficacy in detecting viral entities especially SARS-CoV-2 in the current manuscript which is very flexible and accurate. Detection of virus by rRT-PCR method from nasopharyngeal and oropharyngeal swab is quite satisfactory and accurate one. Furthermore to decrease morbidity and mortality of COVID-19 infection, early and accurate detection of virus as well as public health and infection control measures are urgently required to limit the global threat of this deadly virus.

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Conflict of interest: There is no conflict of interests.

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